

# **A molecular biology approach to protein coupling at a biosensor interface.**

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## **Abstract**

Amino acid residues on the outside of proteins are discussed as potential sites for chemical coupling of proteins to sensor surfaces. This strategy is compared with the use of peptide tags, added to proteins, with an affinity for a particular surface material or chemical structure. Using molecular biology to extend the amino acid protein sequence, in order to include an immobilisation component, is also shown to be suitable for fusion to binding proteins, that can act as the immobilisation partner, so that a compendium of immobilisation strategies is seen to emerge from this common approach of protein engineering.

**Keywords:** Immobilisation; peptide tag; fusion protein; synthetic biology; molecular biology; biosensor.

## **1.Introduction**

The innovation model for biosensors is both application and technology driven. Eg, gene chip/DNA probe technology emerged from the human genome project and was translated to other areas of research and then to medical diagnostics. The original amperometric enzyme electrode concept and the glucose biosensor has achieved a dominant biosensor market, driven by a population where diabetes is increasing. Similarly, other particular assay systems have gained prominence; for example, luciferase, which was discovered in 1962, began to emerge in molecular biology in

the 90s, and is now a core 'reagent' in the development of many new sensor diagnostics. Surface plasmon resonance discovered in 1968 was first incorporated in an immunoassay in 1983. BIAcore first commercialized the technology in 1991 to provide sensing platforms for drug discovery. The diversity of applications emerging from such bio-mechanistic technologies gives some insight into how we might exploit signal generation in/from cell components. However, identification of further signaling systems are only one component required to increase the portfolio of diagnostic information that could be obtained and we are beginning to be able to engineer access to the diversity and selectivity of bio-inspired molecular apparatus with its capacity for analyte recognition, by merging the properties of transduction materials with the capacity of molecular biology.

An important challenge in this mission is the interface between the transducer and biomolecule. Traditional immobilisation methods are broadly categorised into adsorption-based methods [1], entrapment inside a membrane [2], or in a gel/polymer matrix [3], or via cross-linking reagents [4]. These methods do not take the orientation of the biomolecule into account, and regularly end up with the biorecognition molecule showing reduced functionality and poor communication with the underlying transducer [5].

Whereas the historical approach to immobilisation has been to use these traditional chemical methodologies, understanding how biology can be better interfaced with electronic or optical nano- and microsystems, in a platform that can be evolved and reconfigured for detection and diagnosis, is now expanding the biosensor portfolio. This review looks at the evolution of peptide molecular biology immobilisation techniques and considers developments that could further impact the biosensor field. The review begins with reactive amino acids introduced at the surface of a protein target and the strategies that have allowed chemical coupling with various transducer materials (section 2). This method is close to the historical chemical methodologies. In contrast, inspiration from high affinity protein binding partners in

nature offer ways of providing some generic immobilisation methods (section 3) that can be applied to many different systems by fusion of one of the binding partners to the biorecognition element. This also begins to show how our understanding of peptide-protein binding can lead to biorecognition proteins with a fused peptide tag for binding to the transducer surface - all achieved with molecular biology. Peptides and their direct affinity for materials used as transduction surfaces in biosensors therefore expand this concept, with examples of sequences that might be fused to a biorecognition protein (section 4).

## **2. Selected amino acid coupling**

### **2.1 Cysteine coupling**

Due to the low prevalence of cysteine, it can be a natural target for insertion or exchange into a protein's natural sequence. In terms of immobilisation, the choice of cysteine is favoured by easy thiol group chemical activity. Depending on its positioning in the protein, it can therefore be an effective method of controlling protein orientation on the surface. Beissenhirtz *et al* [6] for example, introduced one or two additional cysteine residues in the enzyme, superoxide dismutase, and observed that binding to unmodified gold electrodes could then be achieved, together with direct electron transfer to the electrode, with higher sensitivity towards superoxide radicals compared to conventional cytochrome c.

However, cysteine coupling can also cause inhibition due to opportunist bond formation with non-ideal orientation. For example, Lin *et al* [5] showed that cysteines 14 and 17 of cytochrome c interact with a bare gold electrode inhibiting electron transfer. However, if a C-terminal cysteine tag is introduced on cytochrome c [7], enzyme immobilisation occurs with its binding domain on the opposite side, accessible from solution.

Similarly, when Davis *et al.* [8] engineered a cysteine residue into cytochrome P450<sub>cam</sub>, where the enzyme's haem group was closest to the surface, the enzyme was still catalytically active, and had higher and more ordered coverage of a gold electrode than the wild type enzyme. The activity was also higher, which was attributed to the cysteine being able to orientate the haem group, buried in the protein matrix, so that it was as close as possible to the electrode.

Loechel *et al.* [9] also introduced a cysteine mutation of the residue Y442 in the protein trimethylamine dehydrogenase (TMADH) to couple into the protein's electron transfer pathway to facilitate charge transfer between enzyme and an electrode. However, in this instance the necessary attachment point was in a cleft in the protein which was not accessible to couple directly with an electrode. This required a redox wire, poly-[Fe(5-NH<sub>2</sub>-phen)<sub>3</sub>]<sup>2+</sup>, to join and communicate between enzyme and electrode. Thus, surface modification of an amino acid doesn't necessarily yield a direct match between transducer and protein immobilisation and coupling.

## 2.2 Lysine coupling

Lysine is typically located on the outside of protein because of its polarity and not involved in the catalytic sites. It is often in good abundance, making its amino group an obvious site for further biomolecule interaction and it can be readily and selectively targeted for interaction with activated surfaces (eg -COOH). However, its abundance also inhibits individual site directed immobilisation and orientation of a resultant protein is likely to include any of the surface lysines.

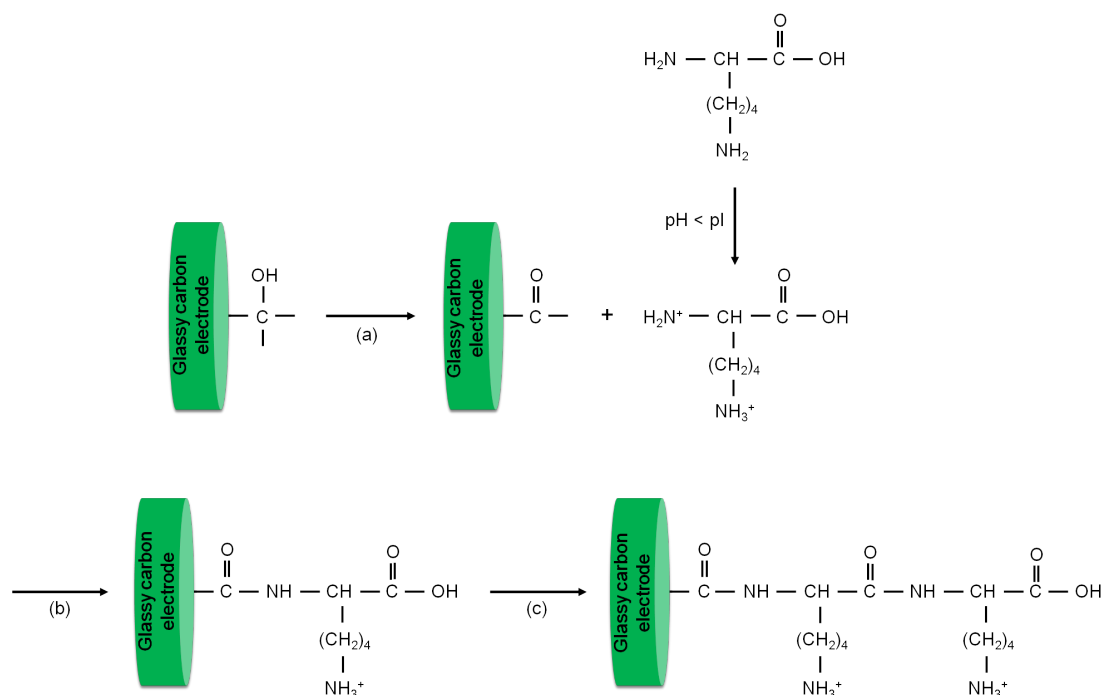
Rather than attempting single amino acid attachment, polylysine could be engineered as a protein tag to increase the likely position for site directed activation, and in principle, it can be electropolymerized on a glassy carbon electrode (GCE) surface by potential cycling (Figure 1), but at rather high potentials where water oxidation is also likely and a part of the surface activation mechanism. Huang *et al.*

[10] have proposed that the polymerisation process involves electrode activation ( $\text{C=O}$ , dependent on the water activation effect) of a GCE, followed by coupling to the protonated  $\text{-NH}_3^+$  of the lysine. This C-N bond formation has also been shown by others and the electrochemical polymerisation method proposed for other aminoacids [11,12]. On the other hand, protonated polylysine itself can be deposited directly on a pre-prepared graphene oxide surface, without electrochemical oxidation, and then provides a good non-specific immobilisation medium for a layer-by-layer type of electrostatic or covalent assembly.

These preparation methods have also been applied in various other types of sensors, not just those involving proteins. Sun *et al.* [13] introduced a DNA sensor using graphene oxide as a platform to immobilize DNA on the surface of polylysine-modified GCE. Polylysine film supported the stabilization of graphene oxide nanosheet through electrostatic attraction on GCE. Due to the high surface area, the authors proposed that an increased amount of probe DNA can be amide bonded, which results in better performance and detection limit of  $1.69 \times 10^{-13}$  M. Similarly, Wang *et al.* [14] immobilized probe DNA on gold nanoparticles, adsorbed on polylysine-modified GCE. Positively charged hexaamineruthenium(III) chloride bound to the negatively charged phosphate back bone of probe DNA on the gold nanoparticle served as a electroactive indicator. Wang demonstrated that this sensor can detect  $3.5 \times 10^{-14}$  M of target DNA. Although these are not polylysine tagged, their outcome suggests that a tagging approach with electrochemical or chemical graphene oxide preparation could also be successful.

Similarly Hua *et al.* [15] applied a polylysine modified GCE in a glucose sensor, where glucose oxidase was immobilized through self-assembly on graphene oxide which had been polylysine-modified. Direct electron transfer was suggested with good electrocatalytic activity in glucose detection of in the range of 0.25 to 5 mM. In the work of Shan *et al.* [16], polylysine was also used, linked to graphene oxide on a

gold electrode. In this instance horseradish peroxidase was the target enzyme and  $\text{H}_2\text{O}_2$  detected with very high sensitivity.



**Figure 1.** Electropolymerization of polylysine film on GCE can be achieved by cyclic voltammetry scanning and reactions including the electrode activation (a), the transfer from L-Lysine molecules to cation molecules (b) and the polymerization of L-Lysine on the electrode surface (c).

Nevertheless, molecular biology can offer other tethering or ‘affinity’ sequences. A major group of affinity tags usually uses a peptide that binds a small ligand linked on a solid support (e.g., his-tags that bind to immobilised metals), while another group includes tags that bind to an immobilised protein partner. These can be useful, for example, for antibody immobilisation and many of these techniques have been developed for protein purification, so that in reviewing peptide tagging for biosensors we can also gain inspiration from this field.

### 2.3 Histidine tag (His-tag)

The use of short histidine stretches or his-tags, usually placed at either the N-

terminus or C-terminus of the required protein, allows advantage to be taken of the interaction between transition metal ions and electron donating histidine groups. Typically, the support requires a ligand modification like nitrilotriacetic acid (NTA) for immobilising transition metal cations such as nickel or copper, to provide an anchoring point for the histidine. The method was developed as an affinity purification tag and is generally thought to lead to only a mild change in protein structure.

Schroper *et al.* [17] considered a histidine tag to control cytochrome c orientation on an Ni-NTA functionalised gold electrode by comparing His-tags added at the N terminus, the C terminus and on both termini of the enzyme. The C terminus His tag, which is close to one the enzyme's main electron transfer pathways, was found to have the most efficient electron transfer with the electrode. Madoz Gurpide *et al.* [18] were able to engineer a surface-exposed  $\alpha$ -helix of ferredoxin:NADP<sup>+</sup> reductase (FNR) with a histidine pair, so that the protein was predicted to be immobilised with the edge of the isoalloxazine FAD ring system perpendicular to the electrode with its electron-exchanging site facing the electrode. They showed that in contrast to other mutants without this predicted orientation advantage, electrocatalytic activity with substrate (NADPH) could be demonstrated. Demin *et al.* [19] genetically modified a deglycosylated glucose oxidase (GOx) with a C terminal hexahistidine tag, which shortened the electron transfer distance and enabled orientated immobilisation on a Cu<sup>2+</sup>-NTA ligand functionlised glassy carbon electrode. This system eliminated the use of mediator for biosensor, and also established a robust fast electrochemical connection between enzyme and electrode.

Mikula *et al.* [20] also used the histidine tagged domain of the receptor for advanced glycation end products (RAGE) linked to a N-acetylcysteamine (NAC) and thiol derivative of diethylene triamine pentaacetic acid (DPTA) modified electrode. The authors concluded that binding of the peptides, A $\beta$ <sub>16-23</sub> and A $\beta$ <sub>1-40</sub>, for early diagnosis of neurodegenerative diseases caused a conformational change of the RAGE

domain which restricted counter ion access. Using  $\text{Cu}^{2+}$  as the probe, this resulted in, a decrease of  $\text{Cu}^{2+}$  redox current. A similar principle was applied in the development of an analytical tool for the screening of interactions between rHIs<sub>6</sub> – Rio1 and compounds that could function as potential kinase inhibitors. [21] In this case, a monolayer of thiol derivative of iminodiacetic acid and NAC were self-assembled on the Au electrode, and the immobilisation of rHIs<sub>6</sub> – Rio1 was achieved by chelating to the  $\text{Cu}^{2+}$  on the electrode surface.

In addition to the thiol group of cysteine, the imidazole group of the histidine, the  $\alpha$ -amino groups of the main chain and the  $\epsilon$ -amino groups of lysine, proteins have various other functional groups that can be covalently bound to modified surfaces under mild conditions. For example, the  $\alpha$ -carboxyl group of the chain; the  $\beta$ - and  $\gamma$ -carboxyl groups of aspartic and glutamic acids; the phenol ring of tyrosine and the indol group of tryptophan are all viable covalent bonding sites. Nevertheless, not all these chemistries give high yields, and additionally the strategies must take resultant activity of the protein into account.

### **3. Coupling via binding proteins**

#### **3.1 Streptavidin-Biotin**

The streptavidin-biotin coupling is possibly one of the most used and versatile immobilization systems to date for many different base biosensors. It is reported to have a high association rate constant ( $k_1 > 10^7 \text{ M}^{-1} \text{ s}^{-1}$ ) and an affinity constant ( $K_D$ ) of  $\sim 10^{-14} \text{ M}$  [22,23]. The two coupling components can be used either way around as anchor and label, but more typically biotin is immobilized using succinimidyl ester chemistry onto an amine-functionalized sensor surface. The NHS–biotin linker may also include a spacer arm such as PEG. Since the dissociation constant associated with the interaction is so low (approximately  $10^{-15} \text{ M}$ ), the binding is very stable, but this also means that the surface is difficult to regenerate for reuse [24].

More recently biotin tagging has been proposed by using the biotin carboxyl carrier



protein (BCCP) [25]. The authors suggest that protein tagging by connecting BCCP to fusion partner proteins would provide a system that could then be directly biotinylated, mediated by the biotin protein ligase (BPL) from the thermophilic archaeon *Sulfolobus tokodaii*. However, instead of requiring that the protein of interest for the assay (antibody, binding protein, enzyme etc) has to be modified with biotin to create the other end of the coupling system, an alternative approach is to use a peptide tag that binds streptavidin directly. For example Streptag II an octapeptide (WSHPQFEK) can be fused with the protein of interest. This is rather an interesting option for chips/surfaces (eg SPR) which might be reused, since the streptag fusion system can be eluted from the surface with a biotin analog. A special advantage of this peptide sequence is that its binding of the tag to streptavidin is not influenced by metal ions, so that it is also suitable for metalloproteins [26].

### **3.2 Glutathione-S -Transferase (GST)**

Another approach to immobilization is the covalent linkage of anti-Glutathione S-transferase (GST) antibody to the sensor chip surface and then using a GST tagged protein for coupling. A range of commercial vectors are available for the expression of GST-fusion proteins, so that cloning, expression, and purification of GST tagged proteins are well documented and straightforward. The GST/anti-GST antibody interaction can be disrupted by washing with glycine/HCl and the anti-GST surface regenerated >200 times, so the system becomes quite versatile. It has been especially popular in SPR devices.

Alternatively, the need for the anti-GST surface can be avoided by achieving connectivity through a glutathione (GSH) – GST affinity bond. Sehr et al. formulated a glutathione S-transferase (GST, 26 kDa) fusion protein system to present a generic capture ELISA using GSH as the capture ligand [27]. They noted that the GST interacted with the  $\gamma$ -glutamyl group of glutathione (GSH) so they linked the sulfhydryl group of GSH to a casein support via sulfosuccinimidyl-4-[p-

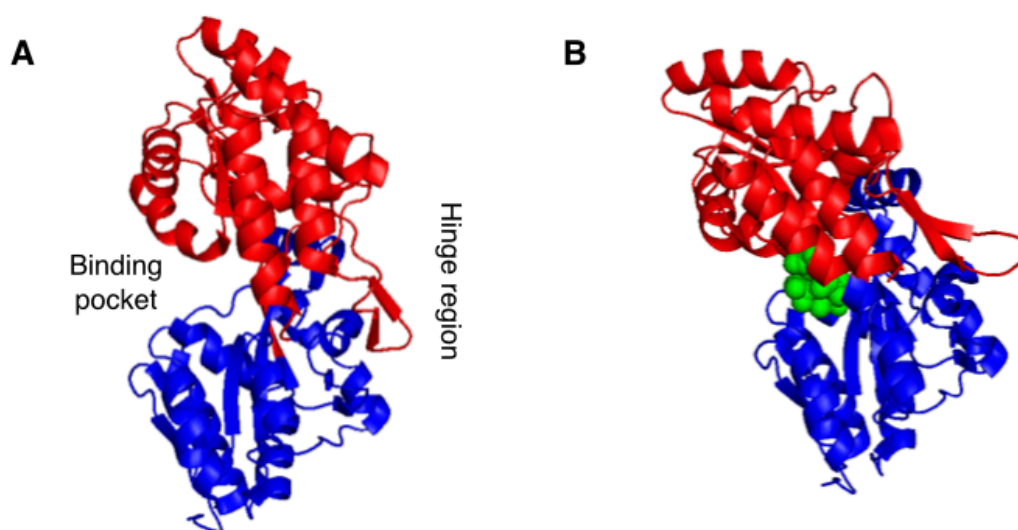
maleimidophenyl] butyrate. The sulfhydryl group is also clearly available for direct self-assembly onto gold. However, Praig and Hall found that in contrast to simple *n*-alkanethiol SAMs, disordered unstable layers were usually formed with GSH. They developed an electrochemical method to form an Au-S bond with the GSH under applied potential >0.2V vs Ag/AgCl and assembled a stable GSH layer on an Au-SPR surface [28]. The resulting layer was able to bind GST specifically at an initial rate of  $\sim 1.2 \times 10^7$  molecules/s, yielding saturation at  $\sim 1.2 \times 10^{10}$  molecules/mm<sup>2</sup> after >5000 s. This is slower than the rate of binding to anti-GST, but produces a stable layer. The authors showed the immobilization of the fusion protein of GST with the plant cyclin-dependent kinase (CDK) Cdc2aAm. The latter belongs to a class of CDKs which interact with cyclins via an evolutionarily conserved 16 amino acids sequence called PSTAIR (EGVPSTAIREISLLKE). The presence and accessibility of the sequence was confirmed using an anti-PSTAIR antibody.

### **3.3 Maltose binding protein (MBP)**

MBP undergoes conformational change upon the presence of maltose from the opened to the closed conformation (Figure 2). This substrate-induced conformational change has been applied widely in the biosensor arena. For example, Fehr *et al.* [29] produced recombinant protein by combining enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP) at each end of MBP. ECFP and EYFP worked as a donor and acceptor chromophore, respectively, and increased fluorescence resonance energy transfer (FRET) when maltose bound to the binding pocket and the distance between ECFP and EYFP was reduced through hinge-bend movement. By using this recombinant protein, a nondestructive dynamic nanosensor has been constructed, which allows better understanding of transportation systems between and within cells by monitoring the concentration of cytosolic maltose.

Similarly, reporter groups, which are conjugated to MBP have been used to generate

currents in the presence of maltose. Benson *et al.* [30] introduced thiol-reactive ruthenium to the cysteine on a mutant MBP, so that the ligand binding site of MBP was designed to point toward the bulk solution, while ruthenium, the reporter group was designed to face the electrode, This then links substrate-induced conformational change and electrochemical signal. With the increased concentration of maltose, the closed form of MBP increased the distance between ruthenium and the gold electrode and the generated signal.



**Figure 2.** Substrate-induced conformational change of maltose binding protein (MBP). Upon the binding of maltose (green spheres), MBP changes its structure from opened form [31] (**A**) to closed form [32] (**B**).

### 3.4 Choline-binding protein (CBP)

With molecular biology techniques becoming increasingly routine, other binding proteins have been identified, also with potential to be both fusion partner for an analyte directing protein and immobilization aid. One promising affinity tag for protein immobilization comes from the C-terminal domain of the choline-binding protein (CBP), LytA amidase, from *Streptococcus pneumoniae* (C-LytA). This originates in

recognition of choline residues of the pneumococcal cell wall. The C-LytA crystal structure has been solved and folding stability increased through protein engineering to enable this system to be used as an affinity tag [33]. To employ this immobilization, a transducer surface needs to be created that presents a choline functionality for interaction. For example, Madoz *et al.* [34] modified a gold electrode with a choline-thiol SAM and assembled a  $\beta$ -galactosidase fused to C-LytA (CLyt- $\beta$ Gal protein) on the surface. Full  $\beta$ -galactosidase activity was retained. Bello-Gil *et al.* have extended this idea so that choline can be presented on a graphite surface [35]. In their example the graphite was functionalized using electrochemical reduction of the diazonium salt formed from 4-aminophenylacetic, similar to the method used for the NTA active surface linking GOx above [19]. However, in this instance activation and esterification of the 4APA carboxylic groups was achieved after attachment to the electrode with a mixture of N,N-diethylethylenediamine (DEAEA), to present a choline-like surface, and ethanolamine (EA) as a spacer, at a ratio of 1:8. The authors report a CLyt- $\beta$ Gal protein loading of 313fmole mm<sup>2</sup> using this method compared with 23fmole mm<sup>2</sup> reported for the same CLyt-bGal on gold [18]. The  $\beta$ Gal oxidized p-aminophenyl  $\beta$ -D-galactopyranoside and the product 4-aminophenol (PAP) could be oxidized at the electrode. Without the CLyt immobilization, the resultant current was circa 3% of the choline immobilized protein.

#### **4. Peptide sequences for coupling**

##### **4.1 FLAG and other antibody selective sequences**

Another approach has been to use the M1 monoclonal antibody (mAb) as an affinity surface. Short affinity tags such as the FLAG hydrophilic octapeptide (DYKDDDDK) will bind M1, although recent studies suggested that a shorter FLAG-related peptide (DYKD) could also be used. This gives rise to the idea that FLAG peptides can be designed as fusion partners with the protein of interest and immobilized to the M1 surface. A FLAG containing plasmid has been constructed to fuse the FLAG motif to

the protein of interest, originally for more accessible purification, Western blot analysis and immunofluorescence assay. Jin *et al.* [36] have taken this a further step by developing a nanovesicle- based bioelectronic nose, combining a single-walled carbon nanotube-based field effect transistor (FET) and human olfactory receptor, containing cell-derived nanovesicles. In this example, the hydrophilic FLAG tag with 3 consecutive aspartate residues hanging on the outside of the vesicle, providing negative charge, may be important for immobilization to the polylysine surface deposited on the FET. This work is also of interest because a second rho-tag (rhodopsin), was also included, targeting the receptor protein on cell membranes and nanovesicles were produced from the construct-expressed human embryonic kidney HEK-293 cells.

Inspiration may also be gained from other sequences that have been devised in the protein purification field such as Softag1 and Softag3. These are small peptides (SLAELLNAGLGGS and TKDPSRVG, respectively) recognized by polyol-selective antibodies, but with potential for interactions with other user-designed surfaces.

## **4.2 Spycatcher**

A rather interesting combination for future consideration in biosensors is the spycatcher system. This is based on the immunoglobulin-like collagen adhesion domain of *Streptococcus pyogenes* (CnaB2). The CnaB2 protein has been divided so that the N-terminal of the protein contains the Lys31 and a peptide residue which includes Asp117 [37]. The key feature of this coupling mechanism is the spontaneous formation of an isopeptide covalent bond between the Lys and Asp. This has been optimized for the purpose of irreversible immobilization to create modified peptide and protein fragments named SpyTag and SpyCatcher, respectively. There are variations of the SpyTag, but in general the original C-terminal sequence of CnaB2 (AHIVMVDA) is extended to increase interaction with

the protein. For example, with SpyTag (AHIVMVDAYKPTK) interaction between the peptide and protein appears to be initiated by the first eight residues of SpyTag that insert into the hydrophobic core of SpyCatcher and the extensive parallel hydrogen bonds with the  $\beta$ -strand of SpyCatcher [38]. The remaining additional amino acids may improve the efficiency and/or stability of the complex. Sun *et al.* has reported covalently cross-linked protein hydrogels prepared by SpyCatcher-SpyTag system. The Spy network acted as artificial extracellular matrix for cell encapsulation, which provides a valuable technique for creation of a biomimetic environment [39]. Chen *et al.* has combined the SpyCatcher-SpyTag interaction with curli amyloid fibrils produced by *E. coli*. These fibrils containing CsgA proteins could help the bacteria to attach to electrode surfaces, and could assemble quantum dots (QDs) with gold nanoparticles (AuNPs) through the SpyCatcher-SpyTag interaction as well as the interaction between anti-FLAG antibodies and FLAG affinity tag. The resulting CsgA<sub>SpyTag</sub> fibrils were bound by the Spy-Catcher conjugated QDs, whereas the CsgA<sub>FLAG</sub> fibrils were bound by AuNPs conjugated secondary anti-FLAG antibodies. Changes in fluorescence lifetime and intensities were obtained by fluorescence-lifetime imaging microscopy (FLIM) for the co-assemblies of QDs and AuNPs compared to the QDs alone [40]. The research lays a foundation for the development of biosensor devices based on fluorescent inorganic materials. The Spy system was also successfully used for protein immobilisation in a “Biofilm Integrated Nanofiber Display” (BIND) system to display protein information to program biofilm, and possess artificial enzymatic, electron transport, and sensing capabilities. The covalent affinity between BIND-SpyTag and SpyCatcher-GFP results in a green fluorescent under confocal microscopy. The successful immobilisation further proved that the SpyTagged biofilm system may be of great importance in the development of biocatalysts and biosensors [41].

### 4.3 Inorganic binding peptides

Many of the examples discussed above require a capture molecule to be bound on the substrate. However, these capture surfaces sometimes reduce the signal measurement efficiency, for example, by extending the distance between the analyte recognition biomolecule and the transducer, resulting in reduced signal. Therefore, in principle, the direct binding of proteins on inorganic materials may be more desirable for efficient measurement. If the protein can be immobilized so that it is directly bound to the transducer surface, then modification of the transducer surface isn't needed. After decades of discovery and development, a huge library containing peptides binding to various materials have been studied. Some bare great potential in photonics, electronics and electrochemical sensing applications (Table 1).

Materials	Sequences	Length
Au	MHGKTQATSGTIQS	14
	VSGSSPDS	8
	WALRRSIRRSY	12
	WAGAKRLVLRRE	12
Pt	PTSTGQA	7
Ti	RKLPGA	6
Ag	AYSSGAPPMPPF	12
	NPSSLFRYLPSD	12
	SLATQPPRTPPV	12
Si	SSKKSGSYSGSKGSKRRIL	19
Cu <sub>2</sub> O	RHTDGLRRIAAR	12
ZnO	RIGHGRQIRKPL	12
	EAHVMHKVAPRP	12
ZnS	NNPMHQN	7
GaAs	AQNPSDNNTHTH	12
	RLELAIPLQSG	12
	TPPRPIQYNHTS	12

CNT	HWKHPSGAWDTL	12
	HWSAWSIRSNQS	12

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**Table 1:** Examples of inorganic binding peptides. The colours indicate the various classes that the individual amino acid falls into. **Yellow** indicates small nonpolar amino acid, **Green** hydrophobic amino acids, **Magenta** polar amino acids, **Red** negatively charged amino acids, and finally **Turquoise** indicate positively charged amino acids.

Peptides are now being discovered with an affinity for abiotic materials by using a combinatorial library approach to screen for potential 'hits' [42]. Sequences have been identified that bind to metals (Au, Ag, Pt [43-47, 56-59]), and oxides /semiconductors (ZnO, Cu<sub>2</sub>O, TiO<sub>2</sub>, SiO<sub>2</sub>, GaN and GaAs [48, 49]). By using these peptides in combination with proteinaceous analytical reagents, immobilization on to surfaces of transducers and components of analytical systems becomes more efficient, without the need for complex chemistries or membranes. For example, Borghei and Hall have reported on a BRET system between Red Fluorescent Protein (RFP) and luciferase [50] that gives a measurement for ATP. When the protein is immobilized on ZnO, energy transfer from the luciferase to the RFP is improved, but immobilization of the protein needs to be directed. Immobilisation to ZnO can be achieved with a His-tag, since the histidine shows an affinity towards Zn<sup>2+</sup> binding, however Yokoo *et al.* [51] used a ZnO-binding peptide (ZnOBP, EAHVMHKVAPRP) [52] for immobilization of recombinant proteins on a ZnO substrate. Although GFP fused with ZnOBP could be weakly immobilized on NiO particles, no GFP-ZnOBP immobilization was observed on TiO<sub>2</sub>, SnO<sub>2</sub>, Fe<sub>2</sub>O<sub>3</sub>, or Al<sub>2</sub>O<sub>3</sub>. By comparing shortened binding sequences, the authors were able to identify that the binding was predominantly driven by an enthalpy change ( $\Delta H$ ) causing variation in  $K_a$ . They concluded that their results indicated that interaction between



the peptide and ZnO surface was via electrostatic binding (At pH 7.5, the surface of ZnO, is positively charged).

Titanium is another useful material for a biosensor surface. Nishida et al have used the peptide described by Sano et al. (RKLPDA: TBP-1 titanium-binding peptide-1) [53] to examine TBP-tagged DNA polymerase (TBP-POL) behaviour on SPR and FET devices [54]. Key residues in this peptide are considered to be the arginine in the first position and the aspartic acid at the fifth position which are proposed to point to the same face and interact with the  $-O^-$  and  $-OH_2^+$  groups respectively, of the oxidized titanium. In the FET model examined, DNA extension was followed with the TBP-POL in the presence of DNA substrate and dNTPs and  $MgCl_2$ . Later in 2005, Sano *et al.* [55] characterized the specificity of TBP-1 and discovered that it binds to Ti, Si and Ag, although mutated versions of it showed less binding activity towards the latter two elements. The finding identifies a general concern with all peptides' capacity for binding specificity and suggests that considerable due diligence is required.

#### **4.4 Au binding peptide**

One challenge with antibody immobilisation is the orientation of the antibody such that its binding sites are available for analyte interaction. A key to this can be to use protein A or G which specifically recognise and bind the Fc portion of antibodies, so that orientation is guaranteed with the Fab sites away from the A or G interaction. Soh *et al.* [56] have proposed a gold binding peptide (AuBP) with three repeats of MHGKTQATSGTIOS for use with protein G whereas Ko *et al.* [57] created the AuBP-Protein A fusion with two repeats of the binding sequence, which showed a high affinity towards gold. Tamerler *et al.* have suggested that hydroxyl and amine groups in methionine, lysine, serine, threonine, glutamine are important for improving

adhesion in gold binding peptides. [58] This provided a method for modification of Au nanoparticles, that could then be attached to a SPR gold chip at a density of  $1 \times 10^9$  particles/cm<sup>2</sup> to offer an antibody immobilization surface. With anti-Salmonella antibodies for detection of *Salmonella typhimurium*, the authors report a 10 fold improvement in detection, compared with classical SPR methodology.

The GBP has also been used successfully in electrochemical biosensors with Au NP-anchored graphene sheets. Yang *et al.* used organophosphorus hydrolase (OPH) as a model enzyme. Key to this particular model system is the chemically modified graphene, which contains the Au NPs, increasing the electroactive area and facilitating electron transfer [59], but also providing an ordered binding point for the GBP (fused with OPH). Whereas the CD showed conformational changes for the OPH enzyme directly associated with the Au-graphene, the secondary structure of GBP-OPH after immobilization onto the surface of Au-graphene was quite similar to that of free GBP-OPH, indicating no conformational changes in GBP-OPH occur during immobilization and resulting in excellent catalytic and electrical properties. The GBP-OPH biosensor system was successfully demonstrated for the detection of paraoxon (2 to 20 mM) [60].

#### **4.5 Si binding peptide**

Biosilification happens naturally in diatoms and sponges, but they adopt very different strategies. Diatoms use silaffins (R5 H<sub>2</sub>N-SSKKSGSYSGSKGSKRRIL-COOH), which are small and heavily modified peptides. Sponges use silicatein, which is homologous to the cysteine protease cathepsin. Both proteins act as structural templates and mechanistic catalysts for the biosilification reaction. [61] Recently, the R5 peptide from the diatom *Cylindrotheca fusiformis* has drawn substantial attention due to its outstanding activity in silica precipitation under mild conditions, and the option to generate genetic fusions of the R5 sequence with a

protein of interest. Such protein-R5 fusions have great potential for immobilisation of proteins on silica, and thus, the development of biosensors. [62,63]

Luckarift *et al.* [64] utilised free R5 peptide as a catalyst for biosilification of tetramethyl orthosilicate (TMOS), to create silica nanospheres entrapping the enzyme butyrylcholinesterase. After immobilisation, 90% of the initial free enzyme activity was retained, compared to 10% retention by using a conventional chemical silification method. Invitski *et al.* [65] adopted a similar method for glucose oxidase (GOx); they entrapped GOx within a conductive TMOS silica matrix, which was achieved by integrating single-walled carbon nanotubes into this biologically derived TMOS silica. Such a strategy not only allows simple integration into biodevices but also presents an opportunity to realise direct electron transfer. Oxidation and reduction peaks at an optimal potential close to that of the FAD/FADH<sub>2</sub> of immobilised GOx were observed from the cyclic voltammetry, and the immobilised GOx was stable for a month without any loss of its enzymatic activity.

Later on, genetically modified R5 with various functional proteins (phosphodiesterase (Pde), organophosphate hydrolase (Opd) and green fluorescent protein (GFP)) were created by Marner *et al.* [66] It was suggested that the loading capacity of the silica formed by the fusion protein R5-Pde was improved 2 fold compared to the loading achieved by mixing of the free R5 and Pde moieties, and 84% of the enzymatic activity was retained as well as its stability being enhanced after entrapment within the silica matrix. The morphology of the silica matrix can be controlled by the number repeats of the R5 units, For example, fusion of Opd with three repeats of R5 could produce smaller silica nanospheres (~ 200nm) compared to the matrix formed by one R5 (~ 500nm).

Recently, the R5 tag has been used in the development of various sensors. Marshall *et al.* [67] created an *in vivo* biosilica-localised ribose sensor that depends on ligand-binding and conformational change to drive FRET-based signalling capabilities. The sensor construct included a bacterial periplasmic ribose binding protein (RBP), flanked by the FRET pair CyPet and YPet, creating a CyPet-RBP-YPet (CRY) sensor cassette, which requires ribose binding and a conformation change by RBP to drive changes in FRET. A silaffin tag was inserted upstream of CRY for localisation in the diatom silica. After transforming the silaffin-CRY sequence into a diatom species, *T. pseudonana*, ribose induced changes in FRET were observed in both living transformed cells and isolated biosilica cell walls. In addition, Nam *et al.* [68] demonstrated silaffin peptides could be used as a signal enhancer for gravimetric biosensors. As a model system, a R5 tagged GFP was immobilised on a gold quartz crystal resonator for quartz crystal microbalances. In this instance however, the R5 tag was of interest as a label and immobilisation used a self-assembly monolayer with a histidine tag. A significant change in resonance frequency was observed when a solution of silicic acid was supplied onto the R5-GFP immobilized surface. Nevertheless, this experiment demonstrates that the R5 concept could be applied both as immobilisation tag or as label, the latter when the protein is tagged with R5, for example in a sandwich assay, and the R5 label is read by inducing biosilification when a silicic acid is applied, consequently increasing the mass and thus amplifying the signal.

#### **4.6 Ag binding peptide**

Silver is another metal that is of considerable interest in SPR sensing and fluorescent imaging. Naik *et al.* [43] discovered a silver binding peptide (AgBP) with the sequence NPSSLFRYLPSD through a combinatorial phage display peptide library and demonstrated the biosynthesis of silver nanoparticles from silver nitrate successfully. Zhang *et al.* [69] further explored the approach by adding chitosan to

the peptide, forming a matrix. As a result, the size and the morphology of the silver nanoparticles are to a certain extent, controllable by the relative ratio of the chitosan and the peptide. The nanoparticles, however, remain largely polydisperse and non-uniform in their shapes. Nonetheless, the ability to synthesise silver nanoparticles using a biocompatible method is particularly interesting in the field of in vitro fluorescent microscopy. Yu *et al.* [70] engineered another AgBP (HDCNKDKHDCNKDKHDCN) and used it to synthesize and stabilise clusters in the cell and obtained fluorescent microscopic images.

#### **4.7 Cu<sub>2</sub>O binding peptide**

Dai *et al.* [71] fused a CN225 sequence (RHTDGLRRIAAR) into a Trali1753 and obtained a DNA and Cu<sub>2</sub>O binding peptide with the ability to assemble Cu<sub>2</sub>O nanoparticles and further organize them into a loopy structure. The extraordinary aspect of this finding is the possibility to insert any inorganic binding motifs into several of the permissive sites of the Trali protein and eventually obtain a multifunctional peptide that has the ability to selectively assemble different materials in solution. This poses great potential in electronic fabrication and sensing applications.

#### **4.8 Carbon Nanotube binding peptides**

Another material that is of great interest in electronics and electrochemistry is carbon nanotube (CNT). Wang *et al.* [72] synthesized two sequences B1 (HWKHPSGAWDTL) and B3 (HWSAWSIRSNOQS) that bind to CNT. The spatial clustering of aromatic and hydrophobic residues in the sequences achieves a folded conformation (one of the low-energy conformations). Such orientation helps the peptide stack on the surface of the nanotube.

#### **4.9 Au-Si binding peptide**

By fusing two peptides with inorganic binding capability together, it is possible to

create an entire class of peptides that have great potential in material synthesis and fabrication. Hnilova *et al.* [73] fused a AuBP (WAGAKRLVLRRE) with a silica binding peptide (QBP) (PPPWLPYMPPWS) using a structural poly-glycine (GGG) spacer, resulting in two forms, AuBP-QBP and QBP-AuBP. Out of the two peptides, QBP-AuBP exhibits great ability to control both the assembly and the synthesis of gold nanostructures on silica substrates. Nochomovitz *et al.* [74] created another one that binds to silica and gold with the sequence of MHGKTQATSGTIQS-GGG-HPPMNASHPHMH. It was used to deposit gold colloids on oxidized silicon wafers. These multifunctional peptides offer better biocompatibility than conventional chemical immobilization methods.

#### **4.10 Au-C binding peptide**

Another example of a bifunctional peptide is one that binds to gold and carbon. The fusion is possible through repetitions of glycine as a linker. Cui *et al.* [75] first discovered a peptide with affinity to graphene (EPLQLKM), and then fused a gold binding peptide to it and created a bifunctional sequence (EPLQLKM-GGGG-AYSSGAPPMPPF). Both the CNTBP and GrapheneBP are rich in aromatic residues that interact favorably with nanotubes and graphene surfaces via  $\pi$ - $\pi$  interactions. The GrapheneBP, in contrast, has alternative hydrophobic and hydrophilic residues which fold to accommodate the planar graphene surfaces. Cui further demonstrated the application of the fused peptide by crafting a TNT sensor around it achieving ultrasensitivity.

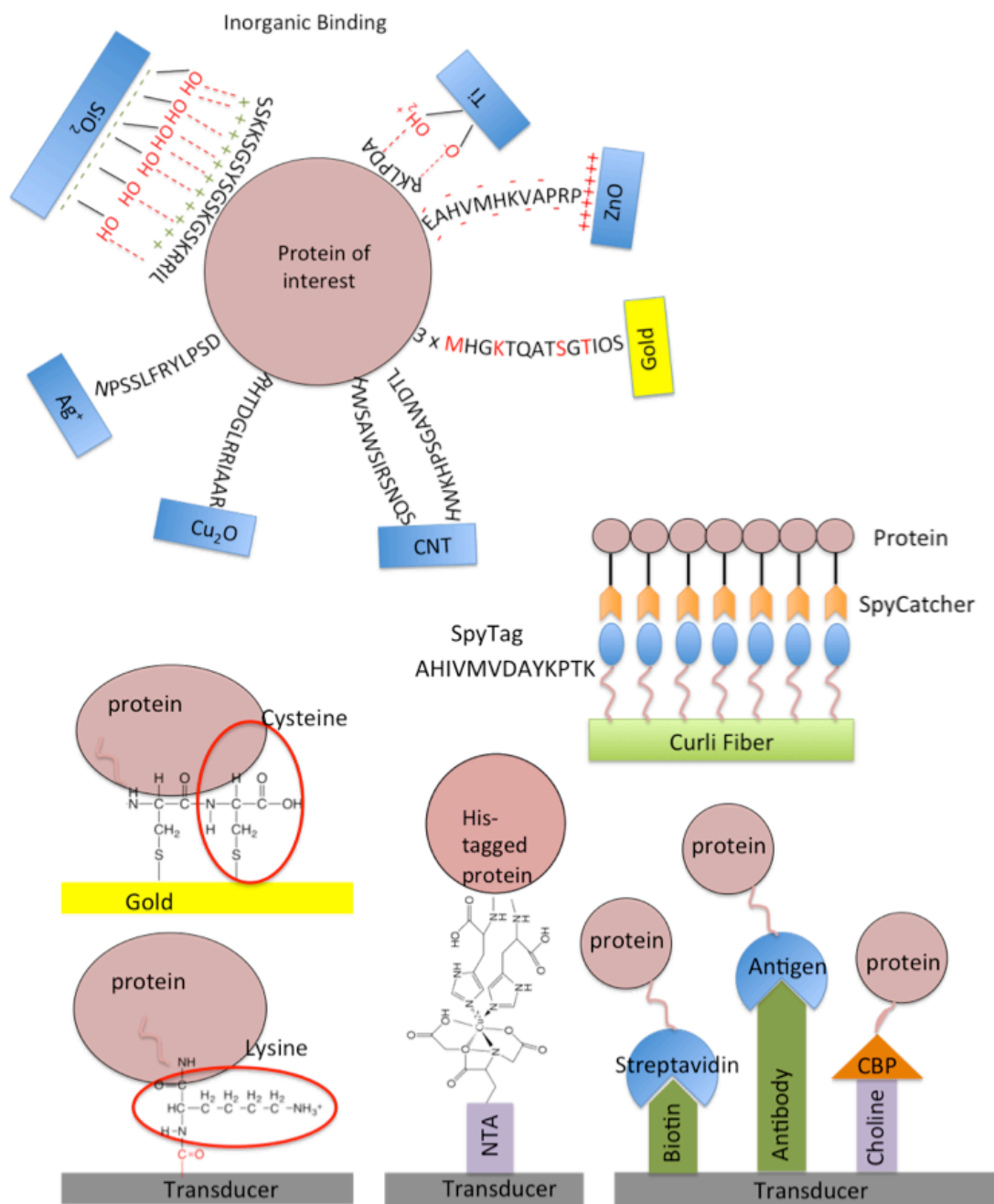


Figure 3: How to immobilize your protein: immobilization strategies using amino acids, peptides and proteins as the binding element.

## 5. Conclusions

It has been seen in this review that by taking a biomimetic approach to immobilisation, couplings ranging from single amino acids and short polypeptide tags to larger binding proteins can be used to achieve a degree of order and stability on a biosensor surface (figure 3). Coupling strategies have invoked many binding and affinity mechanisms whose roots are in other fields. For example, the immunoassay configuration of antibody-antigen also offers a coupling system for the antigen to sensor, via an intermediate antibody. Similarly, peptide sequences from nature with an affinity for a particular material can be used, for example the silaffin sequence that causes silica deposition, will bind to silica when it is part of a biosensor.

So can an immobilization method be selected as a function of the type of protein, i.e. enzyme/ antibody/ binding protein etc.? At present the answer to this important question is probably not. The key criteria in the design process are the matching between the surface material of the sensor and the protein to be immobilised. The front line decision is not whether it is an enzyme or antibody, but where the active site for analyte interaction is and whether molecular biology can be used to place the affinity sequence of choice in a position on that enzyme/antibody to optimise the transduction. The identification of new peptide sequences with affinity for different materials, many having potential as part of a transduction system in a biosensor configuration, and the opportunity to use protein engineering techniques to achieve this coupling, is propagating the use of 'direct' sensor-protein coupling with a fusion or tagged protein.

However, in some tagging cases a chemical modification of the transducer surface is still required. For example, the polyhistidine tag needs a surface that has been modified with a metal chelating agent, and loaded with metal ions, so that a surface can be presented with a divalent ion like  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$  etc.. Nevertheless, even in these



examples, coupling to a surface like ZnS or ZnO<sub>2</sub> offers enough coupling capacity with the histidine for a direct bonding.

The potential advantages of the biomimetic interface approach, comes from the ability to design the orientation of the coupling and achieve it without further chemical modification of the protein and denaturation. Depending on the particular immobilisation model, it can also bring the proteins closer to the transducer, which may improve coupling, for example with redox enzymes such as Cytochrome C, glucose oxidase and TMADH on electrodes, leading to faster electron transfer between enzyme and the electrode. In these cases however, an enzyme specific design is probably required to maximise the coupling efficiency for each protein structure.

In other cases, like the streptavidin-biotin coupling and the glutathione S-transferase (GST) linker a more generic solution applies, which has made these popular choices. These, and other binding proteins, such as maltose binding protein (MBP) and choline binding protein (CBP), are commonly employed in the development of techniques like SPR biosensing devices, where a reproducibly defined base layer can be used, together with many different analyte binding systems and the transduction method needs to respond to the binding event and not an electron transfer or other physicochemical event resulting in some change in state, that can only be detected through intimate contact with the transducer.

Discoveries in peptide properties and development in nanotechnology have further linked the bridge of the interaction between biomolecule and the transducer. This offers new material properties and opportunities for the identification of proteins and peptides with some exquisite affinity or interaction that is likely to further build this immobilisation family in the future.

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